Iron-Quercetin Complex Preconditioning Human Peripheral Blood Mononuclear Cells Accelerates Angiogenic and Wound Healing Efficacy

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Research Article

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Abstract

Background: Cells-based therapy is a highly promising treatment paradigm in ischemic disease due to its ability to repair tissues when implanted into a damaged site. These therapeutic effects have been involving a strong paracrine component resulting from the high levels of bioactive molecules they secrete in response to the local microenvironment. Therefore, the secreted therapeutic can be modulated by preconditioning the cells during *in vitro* culture. Herein, we investigated the potential use of magnetic resonance imaging (MRI) probes “Iron-Quercetin complex” or IronQ for preconditioning peripheral blood mononuclear cells (PBMCs) to expand proangiogenic cells and enhance their secreted therapeutic factors.

Methods: PBMCs obtained from healthy donor blood were cultured in the presence of the Iron-Quercetin complex. Preconditioning-PBMCs differentiated cells were characterized by immunostaining. An enzyme-linked immunosorbent assay to describe the secreted cytokines. In *vitro* migration and tubular formation using human umbilical vein endothelial cells (HUVECs) were completed to investigate the proangiogenic efficacy.

Results: IronQ significantly increased mononuclear progenitor cells' proliferation and differentiation into the spindle-shape-like cells, expressing both hematopoietic and stromal cell markers. The expansion increased the number of colony-forming units (CFU-Hill). The conditioned medium obtained from IronQ-treated PBMCs contained a high level of Interleukin (IL)-8, IL-10, urokinase-type-plasminogen-activator (uPA), matrix metalloproteinases-9 (MMP-9), and tumor necrosis factor-alpha (TNF- α), and augmented migration and capillary network formation of HUVEC and fibroblast cells *in vitro*.

Conclusions: Our study demonstrated that the IronQ-precondition PBMCs protocol could enhance the angiogenic and reparative potential of non-mobilized PBMCs. This protocol can be used as an adjunctive strategy to improve cell therapy's efficacy of PBMCs for ischemic diseases and chronic wound.

Introduction

Cell-based therapy has currently become a focus of regenerative treatment for ischemic diseases and chronic wounds. Clinical improvements have been observed using autologous total mononuclear cells (MNCs) freshly isolated from bone marrow or peripheral blood. These clinical experiences proved that cell-based therapy for vascular regenerations is safe, feasible, and effective [1]. However, the low efficacy of functional cells due to the lower number and dysfunction found in patients has limited its potential as a therapeutic tool [2]. In this study, we consider peripheral blood as a renewable cell source that can be retrieved from a readily accessible body compartment by a low-invasive procedure. However, the peripheral blood mononuclear cells (PBMCs) contain a rarity percentage (<0.01%) of stem/progenitor cells [3, 4]. Moreover, aging or disease also attenuates the numerically and functionally of these functional cells [5]. Therefore, the biggest challenge lies in the enhanced cell expansion and cell

therapeutic potential toward successful and wider clinical application using autologous cells while controlling costs.

Adult peripheral blood-derived stem/progenitor cells are of interest as a potential source of stem cells because they are known to transdifferentiate [6, 7] and comprise the enriched fraction of endothelial progenitor cells (EPCs), which possess superior regenerative efficacy [8]. Moreover, peripheral blood-derived stem/progenitor cells can be obtained from autologous sources without the need for painful bone marrow aspiration. Although some clinical trials have shown potential benefits of peripheral blood-derived stem cells in a patient with ischemic arterial disease [9-12], some controversial issues of the clinical benefits of blood-derived stem cells have emerged. These might be due to the variety of cell isolation protocols and the type of cells administered to patients, such as either enriched progenitor fractions or whole mononuclear cell fractions.

According to the heterogeneity in the peripheral blood mononuclear cells population, our focus is on the critical functions of monocytes/macrophages. Monocytes are precursors of macrophages; monocyte-derived progenitor cells play a crucial role in angiogenesis and regenerative processes. Monocyte-derived cells with spindle shape characteristics named fibrocytes, and endothelial progenitor cells, are pro-angiogenic potential *in vitro* and *in vivo*. Fibrocytes contribute to tissue repair and angiogenesis [13]. They are characterized by the expression of both hematopoietic and stromal cell markers (collagen I, collagen III, CD34, and CD45). Peripheral blood monocyte serves as an enriched source of endothelial progenitor cells. The involvement of EPCs in angiogenesis is well known. EPCs secreted angiogenic factors and differentiation into the endothelial cell [14]. These cells have been shown to affect vascular repair in ischemic hind limb models of vascular injury [15, 16]. Moreover, monocyte cells can also transdifferentiate into endothelial [17], neuronal [18], and mature myeloid cells [19]. Many antigenic markers including, cluster of differentiation 34 (CD34), CD133, CD45, CD31, CD133, CD14, C-X-C Motif Chemokine Receptor 4 (CXCR4), and Vascular endothelial growth factor receptor-2 (VEGF-R2), have been utilized to identify EPC populations [20]. Depend on microenvironment stimulating, macrophages can be polarized into diverse subtypes that play different roles (*i.e.*, the pro-inflammatory M1 phenotype and anti-inflammatory M2 phenotype). Especially, the M2-subtype is playing a central stimulatory role within angiogenesis and tissue repair. These M2-like macrophages secrete an angiogenic factor to regulate vessel organization and vascular surveillance, as evidence by the irregular vessels in the absence of these macrophages [21, 22].

Based on the rarity number of the therapeutic cells, to achieve clinically meaningful cell numbers and the superior quality of cultured cells. A practical approach based on peripheral blood progenitor cells mandates their *ex vivo* expansion is need. Interestingly, dietary compounds, which could promote the angiogenic potential, have been increasingly applied. Quercetin has been extensively studied due to its potential pharmacological properties and beneficial health effects [23]. In particular, quercetin has been reported a beneficial impact on cardioprotective [24]. It also was used as Chinese medicine for the treatment of heart disease [25]. Recent studies have reported that quercetin could enhance the cell proliferation, osteogenic differentiation, and angiogenic factor secretion of healthy rat bone marrow

mesenchymal stem cells (BMSCs) [25]. Moreover, quercetin was also shown to modulate inflammation in humans through mechanisms involving macrophages by enhancing the anti-inflammatory properties of M2 macrophages [26]. However, poor water solubility, chemical instability, and low bioavailability of quercetin can significantly limit its clinical applications [27]. The complexation of quercetin and a large number of metal ions have been reported. It indicates that the biological activities can be improved and increased compared with free quercetin [28].

Recently, we have introduced a novel bi-functional magnetic resonance imaging (MRI) probe, the so-called Iron-Quercetin complex (IronQ) as a new MRI contrast agent for tracking labeled cells. IronQ modulated the cellular characteristic of PBMCs to enrich contained blood-derived spindle-shape-like cells (EPCs) during *in vitro* expansion. Simultaneously, there is the potential to visualize IronQ labeled cells transplant with MR imaging via the paramagnetic properties of IronQ [29]. The Iron-Quercetin complex has some practical advantages over other types of MRI contrast agents when used for clinical purposes. (1) They can be easily prepared without the use of any toxic agents or expensive equipment. (2) They have non-toxicity to cells and can be enriched the circulating progenitor cells in expanded culture. (3) they have the potential to monitor by clinical magnetic resonance imaging. With the use of IronQ with conventional culture technique, blood-derived pro-angiogenic cells can be expanded, increasing their angiogenic potential while tracking IronQ labeled cells via MRI. To minimize the cost of the cell expansion method and the safety profile of the cell tracking procedure, this IronQ was applied to PBMCs. The objective of this study was to examine the effect of the Iron-Quercetin complex on PBMC transforming on the change of surface markers and cytokine and growth factors released. Furthermore, we evaluated the therapeutic potential of expanded cells including, angiogenic and wound healing potentials. The IronQ-preconditioning technique may enhance the isolated unstimulated peripheral blood progenitor cells' therapeutic potential for therapeutic vasculogenesis and tissue regeneration.

Materials And Methods

Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12) were purchased from Caisson Lab (Smithfield, UT, USA). Trypsin-EDTA, fetal bovine serum, penicillin, and streptomycin were purchased from Gibco company (Gibthai, Bangkok, Thailand). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), human insulin, epidermal growth factor, basic fibroblast growth factor, and hydrocortisone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vascular endothelial growth factor (VEGF) was obtained from Thermo Fisher Scientific (Rockford, IL, USA).

Ethics statement

Peripheral blood mononuclear cells (PBMCs) used in this study were obtained from healthy human peripheral blood (age 20 - 40 years, n = 8), all donors provided informed consent. The study was approved

by the Human Research Ethics Committee of the Faculty of Medicine, Chiang Mai University (ref. no. NONE-2560-05052).

Cell isolation and culture

PBMCs were obtained from a buffy coat bag from healthy donor volunteers at the Blood Bank Unit (Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University). PBMCs were isolated from 100 mL of healthy blood donor buffy coat, both male and female. PBMCs were isolated over a Ficoll gradient (Lymphoprep™, Stemcell Technologies, Canada) using the manufacture's protocol.

Mononucleated cell fractions were collected and cultured in RPMI 1640 medium with L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (Manassas, VA, USA). HUVEC were cultured in an endothelial growth medium contained media DMEM/F-12 (10% FBS + 1% penicillin/streptomycin) supplement mix containing epidermal growth factor 5 ng/mL, basic fibroblast growth factor 10 ng/mL, insulin-like growth factor 20 ng/mL, vascular endothelial growth factor 0.5 ng/mL, heparin 22.5 µg/mL, hydrocortisone 0.2 µg/mL, and we defined as EGM medium. L929 Mouse fibroblasts were purchased from American Type Culture Collection (Manassas, VA, USA). L929 were cultured in RPMI 1640 medium with L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin. Cultured all cells in an incubator at 37 °C humidified atmospheres with 5% CO₂.

Iron-Quercetin complex (IronQ) treatment

Fresh PBMCs were seeded at a density of 1×10^6 cells/mL in RPMI 1640 (10% FBS and 1% penicillin/streptomycin) with or without 125 µg/mL Iron-Quercetin complex and cultured at 37 °C humidified atmospheres with 5% CO₂. Experiments were performed after 7, 10, 14 days of culture without subculture or re-feeding. The changed in cell morphology was observed using an inverted microscope (Nikon, ECLIPSE Ts2, Tokyo, Japan).

Cell proliferation assay

The effect of IronQ on cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells (1×10^6 cells/mL) were seed on 24-well plates in the presence of 125 µg/mL IronQ for 1, 3, 5, 7, and 10 days. At the end time point, 200 µL MTT (5 mg/mL) was added to each well and cells were further incubated for four h. Then removed the cultured supernatant and 500 µL of dimethyl sulfoxide (DMSO) was added. The intensity of the formazan solution was determined by measurement of the absorbance at 560 nm using a reader (BioTek™ Eon™ microplate reader, USA).

Flow cytometry analysis

The adherent post-IronQ treated PBMC cells were digested with 0.25% trypsin-EDTA and collected in phosphate buffer saline (PBS). Cell suspensions were stained at 3×10^5 cells in PBS containing 0.1% BSA. Each 100 µL of cell suspension was incubated with IgG isotype controls or with antibodies including

anti-CD34-FITC (Miltenyi Biotec), anti-CD14-FITC (Miltenyi Biotec), anti-CD11b-FITC (Invitrogen), anti-CD45-PE (Invitrogen), anti-CD31-FITC (Life Technologies), anti-CD309-PE (VEGFR-2; eBioscience), and anti-105-APC (eBioscience) for 30 min at 4 °C in the dark. Then, 400 µL of PBS containing 0.1% BSA was added and analyzed using a flow cytometer (Coulter Counter, Epics). Flow cytometric data were analyzed by FlowJo10 software.

Preparation of PBMC conditioned medium

PBMC cells were resuspended in RPMI 1640 medium at a density of 2×10^6 cells/mL and treated with 125 µg/mL IronQ for 10 Days (post-IronQ PBMC-CM). PBMC cells cultured in RPMI 1640 medium without IronQ used as control conditioned medium (Control PBMC-CM). After ten days of incubation, cells were further incubated with serum-free RPMI 1640 medium for 24 h. The cell suspension and conditioned medium were collected and centrifuged to eliminate cells and cellular debris and filtering by passing through a filter 0.22 µm pore size (Millex-Millipore™, Millipore Corporation, MA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The secreted cytokines from PBMCs conditioned medium were collected and investigated by enzyme-linked immunosorbent assay (ELISA). Conditioned medium from PBMCs cultured without IronQ (PBMC Control-CM) or from PBMCs post-IronQ treatment (post-IronQ PBMC-CM) was tested for the presence of IL-8, IL-10, vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase 9 (MMP-9), tumor necrosis factor- α (TNF- α), plasminogen activator inhibitor-1 (PAI-1), and urokinase-type plasminogen activator (uPA) by Human ELISA kit (Invitrogen, USA) following the manufacturer's instructions strictly.

Life cell labeling

The adherent post-IronQ treated PBMC cells were digested with 0.25% trypsin-EDTA and collected in phosphate buffer saline. According to the manufacturer's instructions, cells were labeled with green fluorescence (PKH67 Fluorescent cell linker kits; Sigma-Aldrich, USA).

Tube formation assay on Matrigel

To investigate the angiogenic potential of post-IronQ PBMC cells. Matrigel (Geltrex™, Life Technologies, USA) was used for *in vitro* tube formation assay. Briefly, Matrigel solution was added in 96-well plates at 37 °C for 1 h to allow the matrix solution to solidify. 1×10^4 cells HUVECs were seeded and cultured in 250 µL EGM medium or supernatant culture medium from pre-and-post-IronQ PBMCs. Where indicated, the post-IronQ PBMC cells were harvested and labeled with PKH67 fluorescent cell linker and re-plated (5×10^3 cells/well), and co-cultured with HUVECs on the solidified matrix solution. Cells seeded on Matrigel were incubated at 37 °C. The tubule-like formation was observed under an inverted light microscope. To evaluate the angiogenic capacity, the total length of the tubes formed during the assay was analyzed by Wimasis Image Analysis (Cordoba, Spain). Post-IronQ treated PBMC-labeled cells incorporated into the

tube formation were observed and captured using fluorescence microscopy (Nikon, ECLIPSE Ts2, Tokyo, Japan).

HUVEC Migration with condition medium from post-IronQ treated PBMCs

HUVECs were serum-starved in EGM medium containing 2% FBS for 12 h. Then cells (1×10^5 cells) were seeded at the upper chamber of Transwell inserts of 24-well plates with an 8-mm membrane pore size (Corning, Life Sciences, USA.). The different conditioned medium was added as a chemoattractant in the lower chamber. After 12 h, the medium was aspirated, and the non-migrated cells in the upper surface of each membrane were removed by gently swabbed. Membranes were fixed with methanol and stained with 0.5% crystal violet solution (Sigma-Aldrich, MO, USA). The stained cells were photographed from four random fields of view for each membrane under an inverted microscope (Nikon, ECLIPSE Ts2, Tokyo, Japan).

Scratch wound-induced fibroblast migration assay

L929 fibroblasts cells were grown till they reached full confluence in 6-well plates and incubated overnight in a starvation medium. Cell monolayers were wounded with a sterile 200- μ L pipette tip and washed with phosphate-buffered saline (PBS) to remove the detached cells from the plates. Cells were left untreated or treated with a conditioned medium and kept at 37 °C in a CO₂ incubator for 24 h. The wound gap was observed and photographed using phase-contrast microscopy (Nikon, ECLIPSE Ts2, Tokyo, Japan). The images were then analyzed using the Image J software 1.52v version (National Institutes of Health, USA) to measure the width of the scratch.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD). The statistical analysis of mean comparison was performed by Student's *t*-test using OriginPro version 2018 program. Values of $p \leq 0.05$ were considered statistically significant at the 5% significance level.

Results

PBMCs cultured under IronQ complex results in accelerating proliferative of adherent spindle-shaped cells and number of early outgrowth colonies (CFU-Hill)

Firstly, the appropriate concentration of the Iron-Quercetin complex (IronQ) was determined by cytotoxicity assay in a previous study [29]. We found that 125 μ g/mL of IronQ complex is not toxic to peripheral blood mononuclear cells (PBMCs) when cultured for a long time (i.e., for one month). These concentrations were also available for cell tracking by MRI. Then, PBMCs were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin without adding any specific growth factors, either in the absence (control) or in the presence of 125 μ g/mL IronQ. Spindle shape cells appeared under both

conditions with different characteristics. Under the IronQ condition, cells appear as long spindle cells considerable length of ~100 μm . Inversely, the majority population of attaching cells in the untreated control group appeared a shorter spindle and larger (Fig.1a). The total cell expansion of PBMCs cultured under the IronQ condition compare with the untreated control group was measured. The cell number in culture did not increase in the first 3 days of culture but slightly increased at day 5. The number of cells gradually increased, from 1.3- fold on day 5 and up to 3-fold on day 14 after cells were treated with IronQ while the untreated control group number of cells reached a plateau phase (~1.3-fold) after day 5 of the culture period (Fig.1d). Morphological observation at different time points revealed that IronQ increased the number of adherent cells by almost 90% confluence on Day 14 (Fig. 1b) with an increasing number of the early outgrowth colonies (CFU-Hill) at day 7 post-IronQ treatment (Fig.1c). The CFU-Hill has been described by Hill *et al.*, characterized by the colonies that displayed a central cluster of rounded and flat cells with a radial arrangement of spindle-shaped cells (Fig.1c). These colonies were consistent with the endothelial progenitor cell (EPC) phenotype [30].

Cell population transition and characterization of PBMCs cultured under IronQ complex

To further characterize PBMCs expanded under the IronQ complex condition, surface expression of stem cell markers and markers related to angiogenesis was analyzed using flow cytometry. Based on the scatter diagram, PBMCs post-IronQ treatment (post-IronQ PBMCs) proportionally transition to a large cell population more common than in PBMC untreated control group (pre-IronQ PBMCs) (Fig. 2a). The red lines indicate the cellular-sized gates of lymphocyte and monocyte (R1) and the larger cell (R2). The proportion of each positive cell involved in the whole cells of (R1) and (R2) gates was estimated. The percentage of cells expressing endothelial lineage cells was significantly increased in CD105 and VEGF receptor 2 (VEGFR-2) in PBMCs post-IronQ treatment group whereas, there was no significant difference between the two groups in the number of cells expressing CD31. The percentage of monocytes/macrophages (CD14 and CD11b) was decreased in PBMCs post-IronQ treatment group versus the untreated control group. Besides, we observed a slight decrease in the stem cell marker CD34 in PBMCs post-IronQ treatment group (Fig.2b, c). Altogether, the augmented frequency for VEGFR-2 or CD105 was considerably higher in PBMC post-IronQ cells versus monocytes/macrophages (CD14 and CD11b). These findings indicate that IronQ complex treatment promotes differentiation of circulating progenitor cells in peripheral blood into the pro-angiogenic cells. We also evaluate the dynamic changes of 7 different surface molecules during the culture of PBMCs treated IronQ complex. The result was showed in Fig. 2d. We found that the expression of angiogenic markers CD105 and VEGFR-2 was gradually increasing expressed, whereas expression of CD31 markers remained expressed at variable levels throughout the culture period. Not surprisingly, the pan leukocyte marker CD45 stabilized with culture time, but the stem cell marker CD34 also followed this pattern. The monocyte/macrophage markers were diminished during the culture. Interestingly, we observed that the marker expression was reached the peak on day 10 of the culture period.

PBMCs cultured with IronQ complex secrete vasculogenic, anti-inflammatory, and wound healing factors

Conditioned medium (CM) by PBMCs post-IronQ treatment (post-IronQ PBMCs, at day 10) and untreated control PBMCs (control PBMC-CM) were evaluated for secreted angiogenic, anti-inflammatory, and wound healing factors. It appears that treatment with the IronQ complex stimulates the secreted paracrine factors from PBMCs. These molecules play a critical role in promoted angiogenesis and the wound healing process. The analysis of the amounts of secreted factors by enzyme-linked immunosorbent assay (ELISA). The results (Fig.3) revealed the presence of pro-angiogenic factors including, IL-8, matrix metalloproteinases 9 (MMP-9), and urokinase plasminogen activator (uPA), were significantly higher in post-IronQ PBMC-CM than in the control PBMC-CM group concurrently with the decrease of their inhibitor plasminogen activator inhibitor-1 (PAI-1). Inversely, the VEGF level was lower in post-IronQ PBMC-CM than in the control PBMC-CM. Additionally, the secretion of anti-inflammatory factor IL-10 was significantly higher in the post-IronQ PBMC-CM compared with the control PBMC-CM. Treatment of PBMCs with IronQ complex also promotes the increasing level of tumor necrosis factor-alpha (TNF- α) and monocyte chemoattractant protein-1 (MCP-1) secretion.

PBMCs cultured under the IronQ complex and their conditioned medium shows strong angiogenic properties *in vitro*

The pro-angiogenic potential of PBMCs treated IronQ was investigated by *in vitro* angiogenic assay. Because treatment of PBMCs with IronQ complex secreted proangiogenic factors, we tested the effects of the conditioned medium by post-IronQ PBMCs on the promotion of tube formation in Matrigel of human umbilical vein endothelial cells (HUVECs). HUVECs seeded onto Matrigel in the presence of endothelial growth medium (EGM), condition medium from PBMCs (Control PBMC-CM), or condition medium from IronQ-preconditioning PBMCs (post-IronQ PBMC-CM). HUVECs start to sprout and reorganize into tubular formation very early within 4 h in the presence of condition medium of PBMCs compared to HUVECs cultured in EGM medium alone (Fig. 4a). At 24 h of the time course, HUVECs in all conditions have observed the formation of polygon structures as the endothelial tube network. HUVECs appearance to lose their connections at 60 h in EGM medium, while HUVECs cultured in post-IronQ PBMC-CM still appear as a well-reorganized tube formation (Fig. 4a). We measured cumulative tube length at 24 h after seeding to estimate the stability of the tubular network. The quantitative result revealed a longer cumulative tube length in HUVECs cultured in post-IronQ PBMC-CM. Although, no difference in the total tube length of HUVECs cultured in EGM medium or control PBMCs-CM was observed (Fig. 4c). Moreover, HUVECs in post-IronQ PBMC-CM had significantly increased in the number of tubules per field of view than EGM medium or control PBMC-CM (Fig. 4d). These findings suggested that the treatment of PBMCs with IronQ secreted proangiogenic agents that support the tubular formation of endothelial cells.

To assess the angiogenic potential of the PBMCs treated with IronQ *in vitro*. These PBMCs were labeled with green fluorescence (PKH-67) and then cocultured with HUVEC on Matrigel. IronQ-preconditioning PBMCs colocalized and firmly attached with endothelial tube network. They also participate at the junction point of the endothelial tubular network (Fig. 4b). These results revealed that when cocultured IronQ-preconditioning PBMCs that secreted angiogenic factors, preferential augment the tubular network,

and these post-IronQ PBMCs were able to stabilize the networks. Although these expanded PBMCs did not form a network on their own.

Conditioned medium from PBMCs cultured under the IronQ complex (post-IronQ PBMC-CM) promotes migration of human umbilical cord vein endothelial cells

Since a tube formation involves the migration of HUVEC, we test the chemotactic response of HUVECs to post-IronQ PBMCs secreted factors using a Transwell migration assay. After 24 h, HUVECs migration in response to angiogenic factors secreted by IronQ-preconditioning PBMCs (post-IronQ PBMC-CM) was enhanced three to thirty-fold ($P < 0.05$) over migration in response to the condition medium from control PBMCs (Control PBMC-CM) or the negative control of 0.1% FBS medium alone. And the numbers of migrating HUVECs in the control PBMC-CM were significantly more than those in the negative control group. Not surprisingly, the highest number of migrating cells observed for the positive control group (10% FBS medium) had the greatest-promoting effect. (Fig. 5a, b).

Conditioned medium from PBMCs cultured under the IronQ complex (post-IronQ PBMC-CM) induces migratory ability of fibroblast cells

To investigate whether the PBMCs secreted factors affect the capacity of cell migration on fibroblast. Wound closure migration assay was evaluated. L929 fibroblast cells were grown to confluency, then scratched and treated with ordinary medium or PBMCs secreted factors from condition medium qwwwwwover the time course of 24 h. As shown in figure 6, the post-IronQ PBMC-CM induced a significantly shorter timeframe of L929 fibroblast migration, compared to control PBMC-CM or ordinary medium, at the time point of 1 and 3 h (Fig. 6b). After 9 h, scratched wound closure was almost completely fulfilled in the L929 fibroblast cultures exposed to both of conditioned medium (post-IronQ PBMC-CM and Control PBMC-CM) compared with the ordinary medium, which showed about 75% closure then, and 100% closure after 24 h (Fig. 6a, b). This result indicated that post-IronQ PBMC-CM contained factors that promoted fibroblast cell migration.

Discussion

At present, cell-based therapy has become a great deal of interest in repairing ischemic damage. However, its clinical application has still limited due to the rarity number of regenerative cells. Another challenge facing cell-based therapy is identifying the outcome and effectiveness and real-time *in vivo* monitoring of the transplanted cells [31]. Fortunately, the development of molecular magnetic resonance imaging (MRI) technology provides new approaches for high sensitivity to studying transplanted cells' therapeutic effects by non-invasive dynamic monitoring [32]. Our previous studies investigated the novel MRI contrast agent synthesis and characterization from the Iron-Quercetin complex or IronQ. IronQ is a positive contrast for a T1-weighted MR image. The prominent characteristic of IronQ was the high efficiency of loading into the cells, and the magnetically labeled mononuclear cells were visualized by a clinical 1.5 T MR scanner when the cell quantity was more than 2,000 cells/ μL . Interestingly, the effectiveness of visualizing the IronQ labeled cells were still detectable when the labeled cells were in the

culture for 21 days. It has been shown that IronQ can act as a stimulating agent by favoring the proangiogenic cell differentiation of PBMCs. Moreover, IronQ is highly sensitive and has no toxicity but enhanced the therapeutic efficiency of labeled cells [29]. These results are suggesting that IronQ exerts the most outstanding excellent dual functions in applications of cell-based therapy.

In the present study, we have demonstrated that IronQ preconditioning enhanced *in vitro* function of human peripheral blood mononuclear cells (PBMCs). We found that culturing PBMCs under IronQ conditions were enriched with proangiogenic cells and blood-derived cells with regenerative capacity. Preconditioning PBMCs with IronQ enhanced the therapeutic potential of PBMCs, by secreting the greatest importance cytokines and growth factors supporting revascularization and tissue repair. The use of IronQ preconditioning produced an increase of a spindle-shaped cell type with a surface markers profile characterized by proangiogenic cells, comprising, for example, CD31⁺, CD105⁺, and VEGFR-2⁺ cells. Freshly isolated PBMCs cultured in an endothelial growth factor condition for a short culture period of 4-7 days exhibited a spindle cell-like morphology with a mixed expression profile of CD31, VEGFR-2, CD105, and von Willebrand factor, the so-called “early outgrowth endothelial progenitor cells” (early EPCs) or proangiogenic cells (PACs). Early EPCs contribute to angiogenesis mainly via paracrine signaling mechanisms. Early EPC secreted multiple proangiogenic cytokines and growth factors augment endogenous vessel growth, but they fail to differentiate into endothelial cells [33]. A recent study showed that transplantation of peripheral blood-derived early EPC positive for endothelial markers such as CD31, KDR (VEGFR-2), von Willebrand factor, and CD105 in patients with acute myocardial infarction. Clinical improvements have been observed in these therapeutic cells, including decreased infarct size and an increase in ejection fraction [34, 35]. Culturing PBMCs under the IronQ condition also resulted in enhanced proliferation and PBMCs population transition. The results demonstrated that PBMCs cultured under the condition of IronQ doubled their population number in 7 days of culturing and still increased until Days 10, while the number of PBMCs in the control group progressively decreased with the increased time in culture (Fig. 1, 2). The complex process of tissue repair involves a multistep, including angiogenesis and tissue regeneration, and is tightly controlled by the crosstalk between the cell populations in such a system [36]. Therefore, using a single cell type may be insufficient for successful treatment. There is growing evidence that angiogenesis is a critical process to tissue repair and regeneration. Dashtimoghadam *et al.*, demonstrate that multifunctional cell therapy microcarriers contained mesenchymal stem cells (MSCs), endothelial cells, and vascular endothelial growth factor (VEGF) enhance the regeneration of bone tissue *in vivo* [37]. The new blood vessel formation is a prominent process during tissue repairing the damage. Recently, the study showed that combined use of stem cells and endothelial cells harness the therapeutic effect on cardiac damage regeneration than using stem cells or endothelial cells alone [38]. All together suggest that the pro-angiogenesis condition is a critical factor for promoting tissue regeneration.

Our study adopted a heterogeneous cell mixture model of mononuclear cells to allow for crosstalk between all components of PBMCs. We hypothesized that IronQ would stimulate the mononuclear cells to amplify cytokine signals and crosstalk between the significant cell population in such a system. The

cytokine secreted from PBMCs treatment with IronQ are well known for their capacity to promote angiogenesis such as IL-8, MCP-1, uPA, MMP-9, and VEGF. These angiogenic factors are potent secreted from various monocyte-derived angiogenic cells, including EPCs, pericytes, monocytes, and macrophages cells [39-41]. Also, IronQ can stimulate mononuclear cells to produce the cytokine that contributes to regulating T cell differentiation, such as IL-10. This cytokine produced by M2 macrophages and induces Th2 and Treg lymphocyte functions [42]. M2 macrophages ameliorate anti-inflammatory and immune-suppressive phenotypes, and they promote angiogenesis and tissue repair. It is reported that intracellular iron status act as in modulation of macrophage plasticity and polarization. A recent study revealed the influence of iron on innate immune and macrophage polarization *in vivo* and *in vitro*. The researchers found that an iron-rich status promotes M2 subtype macrophage and impaired M1 subtype activated response to LPS-induced pro-inflammatory [43]. Therefore, IronQ that contained iron might stimulate and load intracellular mononuclear cells resulting in PBMCs transform into alternative M2 macrophage cells. A result of cytokine secreted by M2 macrophage (IL-10) regulates T lymphocyte transform phenotypically polarized into their respective regenerative subsets (Th2 and Treg lymphocytes). Interestingly, IronQ treatment can induce PBMCs to expand proangiogenic cells and change blood cells into the cells responsible for vascular and tissue regeneration. Through the IronQ treatment results of various combinations of therapeutic cells and secreted cytokines, we could conclude that cytokines play a synergistic role in the priming process. However, future experiments may involve the closer examination of responsible factors and cells to IronQ treatment for the generation of therapeutic cells.

Growing evidence from recent studies strongly suggests highlighting the paracrine effect of the transplanted cells in cell therapy [44]. In our research, IronQ preconditioning PBMCs could promote angiogenesis and tissue repair mainly via a paracrine manner. The conditioned medium analysis obtained from post-IronQ treated PBMCs contained a more incredible amount of several key proangiogenic cytokines, such as IL-8, uPA, MMP-9, MCP-1, TNF-alpha. Importantly, IL-8, which is a well-known proangiogenic cytokine and plays a significant role in angiogenesis. Regarding their association with angiogenesis, the therapeutic target on IL-8 is currently under research for cancer therapy [45]. The angiogenic property of IL-8 is involved in endothelial cell proliferation and capillary tube organization [46]. Also, urokinase plasminogen activator (uPA), which increased more than 10-fold in post-IronQ PBMCs conditioned medium, plays a role in angiogenesis through proteolytic degradation of the extracellular matrix that facilitates the subsequent proliferation and migration of endothelial cells [47]. This finding indicated that post-IronQ treated PBMCs were higher secreted proangiogenic factors. They may be reflected in our observation of enhanced tube formation and migration of HUVEC after treatment with condition medium from post-IronQ treated PBMCs. However, VEGF, one of the main proangiogenic growth factors, was not elevated but reduced in post-IronQ treated PBMCs, relative to control PBMCs. A negative feedback mechanism might explain the reduction of VEGF level through a high secreted TNF-alpha in post-IronQ treated PBMCs conditioned medium.

Tumor necrosis factor-alpha (TNF- α) is a secretory product of activated macrophages, a crucial proinflammatory mediator. In our finding, the secreted level of TNF- α slightly elevated in post-IronQ treated PBMCs, relative to control PBMCs. This finding indicates that post-IronQ treated PBMCs may

bring proinflammatory cell populations more than control PBMCs. However, TNF- α has been implicated in angiogenesis during inflammation, wound repair, and tumor growth [48]. It has been reported that TNF- α mediates crosstalk between macrophages and ECs at sites of inflammation and enhanced and temporally regulated angiogenic sprouting begins [49]. These might be reflecting in our observed very early endothelial sprouting of HUVEC culture in Matrigel assay with the presence of culture medium from post-IronQ treated PBMCs, relative to conventional EGM medium. However, TNF- α may support angiogenesis via induced expression of proangiogenic genes, such as VEGFR-2, while blocking signaling through VEGFR-2. It results in delayed angiogenesis in the acute phase of inflammatory response. Therefore, TNF- α can exert pro- or anti-angiogenic effects depending on its expression during the angiogenic process [49]. Furthermore, TNF- α also is a potent fibroblast chemoattractant and stimulates fibroblasts proliferation and the local application of TNF- α in collagen-based biomaterials can improve promote healing of injured tissue [49-51]. Herein, IronQ-treated PBMCs secreted a higher level of TNF- α . This phenomenon may be reflected in the findings of enhanced migration of L929 fibroblast after cultured in post-IronQ treated PBMCs conditioned medium (Fig. 6). Moreover, in our study, the secreted of MMP-9, which playing a critical role in neovascularization and tissue remodeling for anti-fibrosis, were elevated in the post-IronQ treated PBMCs conditioned medium. Taken together, TNF- α , through its actions with MMPs, may play a role in improved wound healing. The recruitment and activation of monocytes/macrophages within the ischemic tissues are essential for the tissue repair process [52]. These secretory cytokines are known to be important in wound healing [53, 54]. These results suggest that PBMCs treatment with IronQ complex promotes PBMCs to secrete high levels of growth factors and cytokines that are important for angiogenesis and the wound healing process. Following ischemic damage, the most frequently observed chemokines is monocyte chemoattractant protein-1 (MCP-1). The MCP-1 is associated with monocyte recruitment and has been considered as one crucial proangiogenic factor [55]. A recent study in the hindlimb ischemic model has shown the excellent benefit of administering exogenous MCP-1 to increase blood flow to the ischemic tissue via the increase in monocyte/macrophage recruitment and augment the development of collateral neovascularization [56]. Furthermore, MCP-1 has been shown to mobilize and transdifferentiate the mononuclear monocyte lineage cells into endothelial-like cells [57].

The non-healing phenotype of chronic wounds is characterized by a lack of vascularization and wound re-epithelialization [58]. The wound healing process involves a multistep, including inflammation, angiogenesis, and tissue regeneration of the skin. These complex processes are tightly controlled by the interplay of different cell types in the wounded tissues, including inflammatory cells, fibroblasts, keratinocytes, and endothelial cells [59, 60]. IronQ-treated PBMCs significantly enhance angiogenesis (tube formation) by secreting growth factors and cytokines. IronQ-preconditioning PBMCs may result in a more favorable vascular regeneration condition or tissue repair because of the orchestration of multiple cytokines' dynamic expression and growth factors. The latter effects were presumably the result of IronQ-preconditioning PBMCs effects by accelerated phenotypes of macrophages and T lymphocytes and proangiogenic cells. These secreted agents also effectively induced migration of fibroblast and

endothelial cells (HUVECs). Therefore, this efficacious outcome of IronQ-preconditioning PBMCs provides a proof of principle for the therapeutic efficacy of IronQ-preconditioning PBMCs.

Previous publications strongly support our interest in the clinical application of IronQ-treated PBMC to treat hard-to-heal wounds or chronic skin defects (ulcers, diabetic wounds...). During a clinical first clinical prospective phase I about using autologous secretome of PBMCs in humans, Simader et al. have shown that such a treatment was safe and well-tolerated [61]. Recently, Gugerell et al. announce the success in the first clinical phase II trial of using the topically administered cell-free secretome of peripheral blood mononuclear cells (APOSEC) in patients suffering from diabetic foot ulcers [62]. Scatena et al. reported that PBMCs showed a favorable clinical outcome at two years follow-up in patients with diabetic foot ulcers and no-option critical limb ischemia, significantly reducing the amputation rate and improving survival and wound healing [63].

Other teams have demonstrated animal models that expanded PBMCs as a relevant therapeutic vector for chronic skin wound healing. Tanaka et al. have shown that ex vivo-expanded PBMCs, and transplanted into euglycemic and diabetic wounds in mice, led to wound closure acceleration, maturation, and vascularization [64]. Comparative effects were observed by Mildner et al., who used secretome obtained from PBMC to improve wound healing response in a murine model [65]. In pigs, the transplantation of endothelial progenitor cells (EPCs) was used to promote wound closure and angiogenesis. This work was published by Kado et al., who were able to enrich PBMC with EPCs and transplanted them into wounds performed on cyclosporine-immunosuppressed pigs [66]. Therefore, the combination of these data from the literature and our results contribute to consider IronQ-preconditioning PBMCs as a promising approach for treating chronic wounds.

Conclusions

The novel technique, IronQ-preconditioning PBMCs, investigated in this study, could be an alternative method for cell expansion and therapy for ischemic diseases and chronic wounds. It has the advantage of using paramagnetic agents to expand the limited number of progenitor cells from non-mobilized PBMCs without the addition of growth factors and serve as the magnetic label for MRI at the same time. However, further study is the warranty for a better understanding of the mechanism under the effect of IronQ on EPCs.

Abbreviations

CFU: colony-forming units; PBMCs: peripheral blood mononuclear cells; MRI: magnetic resonance imaging; HUVEC: human umbilical vein endothelial cells; EPC: endothelial progenitor cell; uPA: urokinase plasminogen activator; TNF- α : tumor necrosis factor-alpha; FBS: fetal bovine serum; CM: conditioned medium; MMP-9: matrix metalloproteinases-9; IronQ: Iron-Quercetin complex; MNC: mononuclear cell; VEGF: vascular endothelial growth factor; VEGFR-2: vascular endothelial growth factor receptor-2; MCP-1:

monocyte chemoattractant protein-1; EGM: endothelial growth medium; FOV: field of view; MSC: mesenchymal stem cell; PACs: proangiogenic cells; PAI-1: plasminogen activator inhibitor-1

BSA: bovine serum albumin; EGF: epidermal growth factor; bFGF: basic fibroblast growth factor; FITC: fluorescein isothiocyanate; APC: allophycocyanin; PE: phycoerythrin; DMSO: dimethyl sulfoxide.

Declarations

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

JK designed methodology, performed investigation and formal analysis, and prepared original draft of the manuscript. NA performed investigation and formal analysis and edited the manuscript. NL performed visualization. GR performed visualization and edited the manuscript. JV visualization and edited the manuscript. ND conceptualized the study, supervised for the study, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate.

Peripheral blood mononuclear cells (PBMCs) used in this study were obtained from buffy coat of healthy young donor's volunteers in Blood Bank Section, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University after their written informed consent. The study was approved by the Human Research Ethics Committee of the Faculty of Medicine, Chiang Mai University (ref. no. NONE-2560-05052).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Prasad K, Sharma A, Garg A, Mohanty S, Bhatnagar S, Johri S, et al. InveST Study Group. Intravenous autologous bone marrow mononuclear stem cell therapy for ischemic stroke: a multicentric, randomized trial. *Stroke*. 2014 Dec;45(12):3618-3624. doi: 10.1161/strokeaha.114.007028.
2. Pittenger MF, Discher DE, Péault BM, Phinney DG, Hare JM, Caplan AI. Mesenchymal stem cell perspective: cell biology to clinical progress. *NPJ Regen Med*. 2019;4:22. doi: 10.1038/s41536-019-0083-6.
3. Hénon P. Key success factors for regenerative medicine in acquired heart diseases. *Stem Cell Rev Rep*. 2020;16:441-58. doi:10.1007/s12015-020-09961-0.
4. Elfenbein GJ, Sackstein R. Primed marrow for autologous and allogeneic transplantation: a review comparing primed marrow to mobilized blood and steady-state marrow. *Exp Hematol*. 2004;32:327-39. doi:10.1016/j.exphem.2004.01.010.
5. Lee PS, Poh KK. Endothelial progenitor cells in cardiovascular diseases. *World J Stem Cells*. 2014;6:355-66. doi:10.4252/wjsc.v6.i3.355.
6. Körbling M, Katz RL, Khanna A, Ruifrok AC, Rondon G, Albitar M, et al., Estrov Z. Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. *N Engl J Med*. 2002;346:738-46. doi:10.1056/nejmoa3461002.
7. Yeh ET, Zhang S, Wu HD, Korbling M, Willerson JT, Estrov Z. Transdifferentiation of human peripheral blood CD34⁺-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells *in vivo*. *Circulation*. 2003;108:2070–3. doi:10.1161/01.CIR.0000099501.52718.70.
8. Chong MS, Ng WK, Chan JK. Concise Review: Endothelial progenitor cells in regenerative medicine: applications and challenges. *Stem Cells Transl Med*. 2016;5:530-8. doi:10.5966/sctm.2015-0227.

9. Perin EC, Silva GV. Cell-based therapy for chronic ischemic heart disease—a clinical perspective. *Cardiovasc Ther*. 2011;29:211-7. doi:10.1111/j.1755-5922.2010.00214.x.
10. Assmus B, Schächinger V, Teupe C, Britten M, Lehmann R, Döbert N, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation*. 2002;106:3009-17. doi:10.1161/01.cir.0000043246.74879.cd.
11. Dong Z, Chen B, Fu W, Wang Y, Guo D, Wei Z, et al. Transplantation of purified CD34⁺ cells in the treatment of critical limb ischemia. *J Vasc Surg*. 2013;58:404-11. doi:10.1016/j.jvs.2013.01.037.
12. Kinoshita M, Fujita Y, Katayama M, Baba R, Shibakawa M, Yoshikawa K, et al. Long-term clinical outcome after intramuscular transplantation of granulocyte colony stimulating factor mobilized CD34 positive cells in patients with critical limb ischemia. *Atherosclerosis*. 2012;224:440-5. doi:10.1016/j.atherosclerosis.2012.07.031.
13. Li J, Tan H, Wang X, Li Y, Samuelson L, Li X, et al. Circulating fibrocytes stabilize blood vessels during angiogenesis in a paracrine manner. *Am J Pathol*. 2014;184(2):556-71. doi:10.1016/j.ajpath.2013.10.021.
14. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation*. 2003;107:1164-9. doi:10.1161/01.cir.0000058702.69484.a0.
15. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, et al. Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet*. 2002;360:427-35. doi:10.1016/S0140-6736(02)09670-8.
16. Kang KT, Lin RZ, Kuppermann D, Melero-Martin JM, Bischoff J. Endothelial colony forming cells and mesenchymal progenitor cells form blood vessels and increase blood flow in ischemic muscle. *Sci Rep*. 2017;7:770. doi:10.1038/s41598-017-00809-1.
17. Tso C, Rye KA, Barter P. Phenotypic and functional changes in blood monocytes following adherence to endothelium. *PLoS One*. 2012;7:e37091. doi:10.1371/journal.pone.0037091.
18. Kodama H, Inoue T, Watanabe R, Yasutomi D, Kawakami Y, Ogawa S, et al. Neurogenic potential of progenitors derived from human circulating CD14⁺ monocytes. *Immunol Cell Biol*. 2006;84:209-17. doi:10.1111/j.1440-1711.2006.01424.x.
19. Weiskopf K, Schnorr PJ, Pang WW, Chao MP, Chhabra A, Seita J, et al. Myeloid cell origins, differentiation, and clinical implications. *Microbiol Spectr*. 2016;4:10. doi:10.1128/microbiolspec.MCHD-0031-2016.

20. Cheng CC, Chang SJ, Chueh YN, Huang TS, Huang PH, Cheng SM, et al. Distinct angiogenesis roles and surface markers of early and late endothelial progenitor cells revealed by functional group analyses. *BMC Genomics*. 2013;14:182. doi:10.1186/1471-2164-14-182.
21. Corliss BA, Azimi MS, Munson JM, Peirce SM, Murfee WL. Macrophages: An inflammatory link between angiogenesis and lymphangiogenesis. *Microcirculation*. 2016;23:95-121. doi:10.1111/micc.12259.
22. DeFalco T, Bhattacharya I, Williams AV, Sams DM, Capel B. Yolk-sac-derived macrophages regulate fetal testis vascularization and morphogenesis. *Proc Natl Acad Sci U S A*. 2014;111: E2384–93. doi:10.1073/pnas.1400057111.
23. Wong SK, Chin KY, Ima-Nirwana S. Quercetin as an agent for protecting the bone: a review of the current evidence. *Int J Mol Sci*. 2020;21:6448. doi:10.3390/ijms21176448.
24. Li M, Jiang Y, Jing W, Sun B, Miao C, Ren L. Quercetin provides greater cardioprotective effect than its glycoside derivative rutin on isoproterenol-induced cardiac fibrosis in the rat. *Can J Physiol Pharmacol*. 2013;91:951-9. doi:10.1139/cjpp-2012-0432.
25. Zhou Y, Wu Y, Jiang X, Zhang X, Xia L, Lin K, et al. The effect of quercetin on the osteogenesis differentiation and angiogenic factor expression of bone marrow-derived mesenchymal stem cells. *PLoS One*. 2015;10:e0129605. doi:10.1371/journal.pone.0129605.
26. Derlindati E, Dall'Asta M, Ardigò D, Brighenti F, Zavaroni I, Crozier A, et al. Quercetin-3-O-glucuronide affects the gene expression profile of M1 and M2a human macrophages exhibiting anti-inflammatory effects. *Food Funct*. 2012;3:1144-52. doi:10.1039/c2fo30127j
27. Tran TH, Guo Y, Song D, Bruno RS, Lu X. Quercetin-containing self-nanoemulsifying drug delivery system for improving oral bioavailability. *J Pharm Sci*. 2014;103:840-52. doi:10.1002/jps.23858.
28. Zhou J, Wang LF, Wang JY, Tang N. Synthesis, characterization, antioxidative and antitumor activities of solid quercetin rare earth (III) complexes. *J Inorg Biochem*. 2001;83:41-8. doi:10.1016/s0162-0134(00)00128-8.
29. Papan P, Kantapan J, Sangthong P, Meepowpan P, Dechsupa N. Iron (III)-Quercetin Complex: synthesis, physicochemical characterization, and MRI cell tracking toward potential applications in regenerative medicine. *Contrast Media Mol Imaging*. 2020;2020:8877862. doi:10.1155/2020/8877862.
30. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med*. 2003;348:593-600. doi:10.1056/NEJMoa022287.
31. Heathman TR, Nienow AW, McCall MJ, Coopman K, Kara B, Hewitt CJ. The translation of cell-based therapies: clinical landscape and manufacturing challenges. *Regen Med*. 2015;10:49-64.

doi:10.2217/rme.14.73.

32. Ngen EJ, Artemov D. Advances in monitoring cell-based therapies with magnetic resonance imaging: Future perspectives. *Int J Mol Sci.* 2017;18:198. doi:10.3390/ijms18010198.
33. Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol.* 2008;28:1584-95. doi:10.1161/ATVBAHA.107.155960.
34. Britten MB, Abolmaali ND, Assmus B, Lehmann R, Honold J, Schmitt J, et al. Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): mechanistic insights from serial contrast-enhanced magnetic resonance imaging. *Circulation.* 2003;108:2212-8. doi:10.1161/01.CIR.0000095788.78169.AF.
35. Schächinger V, Assmus B, Britten MB, Honold J, Lehmann R, Teupe C, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. *J Am Coll Cardiol.* 2004;44:1690-9. doi:10.1016/j.jacc.2004.08.014.
36. Ahn A, Frishman WH, Gutwein A, Passeri J, Nelson M. Therapeutic angiogenesis: a new treatment approach for ischemic heart disease-part I. *Cardiol Rev.* 2008;16:163-71. doi:10.1097/CRD.0b013e3181620e3b.
37. Dashtimoghadam E, Fahimipour F, Tongas N, Tayebi L. Microfluidic fabrication of microcarriers with sequential delivery of VEGF and BMP-2 for bone regeneration. *Sci Rep.* 2020;10:11764. doi:10.1038/s41598-020-68221-w.
38. Suuronen EJ, Price J, Veinot JP, Ascah K, Kapila V, Guo XW, et al. Comparative effects of mesenchymal progenitor cells, endothelial progenitor cells, or their combination on myocardial infarct regeneration and cardiac function. *J Thorac Cardiovasc Surg.* 2007;134:1249-58. doi:10.1016/j.jtcvs.2007.07.028.
39. Rivera LB, Bergers G. Intertwined regulation of angiogenesis and immunity by myeloid cells. *Trends Immunol.* 2015;36:240-9. doi:10.1016/j.it.2015.02.005.
40. Yoon CH, Hur J, Park KW, Kim JH, Lee CS, Oh IY, et al. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases. *Circulation.* 2005;112:1618-27. doi:10.1161/CIRCULATIONAHA.104.503433.
41. Li J, Tan H, Wang X, Li Y, Samuelson L, Li X, et al. Circulating fibrocytes stabilize blood vessels during angiogenesis in a paracrine manner. *Am J Pathol.* 2014;184:556-71. doi:10.1016/j.ajpath.2013.10.021.
42. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol.* 2010;11:889-96. doi:10.1038/ni.1937.

43. Agoro R, Taleb M, Quesniaux VFJ, Mura C. Cell iron status influences macrophage polarization. *PLoS One*. 2018;13:e0196921. doi:10.1371/journal.pone.0196921.
44. Liang X, Ding Y, Zhang Y, Tse HF, Lian Q. Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant*. 2014;23:1045-59. doi:10.3727/096368913X667709.
45. Ning Y, Manegold PC, Hong YK, Zhang W, Pohl A, Lurje G, et al. Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models. *Int J Cancer*. 2011;128:2038-49. doi:10.1002/ijc.25562.
46. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol*. 2003;170:3369-76. doi:10.4049/jimmunol.170.6.3369.
47. Oh CW, Hoover-Plow J, Plow EF. The role of plasminogen in angiogenesis in vivo. *J Thromb Haemost*. 2003;1:1683-7. doi:10.1046/j.1538-7836.2003.00182.x.
48. Hsu T, Nguyen-Tran HH, Trojanowska M. Active roles of dysfunctional vascular endothelium in fibrosis and cancer. *J Biomed Sci*. 2019;26:86. doi:10.1186/s12929-019-0580-3.
49. Sainson RC, Johnston DA, Chu HC, Holderfield MT, Nakatsu MN, Crampton SP, et al. TNF primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype. *Blood*. 2008;111:4997-5007. doi:10.1182/blood-2007-08-108597.
50. Chen X, Thibeault SL. Role of tumor necrosis factor-alpha in wound repair in human vocal fold fibroblasts. *Laryngoscope*. 2010;120:1819-25. doi:10.1002/lary.21037.
51. Tottoli EM, Dorati R, Genta I, Chiesa E, Pisani S, Conti B. Skin wound healing process and new emerging technologies for skin wound care and regeneration. *Pharmaceutics*. 2020;12:735. doi:10.3390/pharmaceutics12080735.
52. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol*. 2011;11:762-74. doi:10.1038/nri3070.
53. Siqueira MF, Li J, Chehab L, Desta T, Chino T, Krothpali N, et al. Impaired wound healing in mouse models of diabetes is mediated by TNF-alpha dysregulation and associated with enhanced activation of fork head box O1 (FOXO1). *Diabetologia*. 2010;53:378-88. doi:10.1007/s00125-009-1529-y.
54. Wood S, Jayaraman V, Huelsmann EJ, Bonish B, Burgad D, Sivaramakrishnan G, et al. Pro-inflammatory chemokine CCL2 (MCP-1) promotes healing in diabetic wounds by restoring the macrophage response. *PLoS One*. 2014;9: e91574. doi:10.1371/journal.pone.0091574.

55. Niu J, Azfer A, Zhelyabovska O, Fatma S, Kolattukudy PE. Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). *J Biol Chem*. 2008; 283:14542-51. doi:10.1074/jbc.M802139200.
56. Voskuil M, van Royen N, Hoefer IE, Seidler R, Guth BD, Bode C, et al. Modulation of collateral artery growth in a porcine hindlimb ligation model using MCP-1. *Am J Physiol Heart Circ Physiol*. 2003;284:H1422-8. doi:10.1152/ajpheart.00506.2002.
57. Niu J, Wang K, Zhelyabovska O, Saad Y, Kolattukudy PE. MCP-1-induced protein promotes endothelial-like and angiogenic properties in human bone marrow monocytic cells. *J Pharmacol Exp Ther*. 2013;347:288-97. doi:10.1124/jpet.113.207316.
58. Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med* 1999;341:738–46. doi:10.1056/NEJM199909023411006.
59. Kondo T, Ishida Y. Molecular pathology of wound healing. *Forensic Sci Int*. 2010;203:93-8. doi:10.1016/j.forsciint.2010.07.004.
60. Frykberg RG, Banks J. Challenges in the Treatment of Chronic Wounds. *Adv Wound Care (New Rochelle)*. 2015;4:560-82. doi:10.1089/wound.2015.0635.
61. Simader E, Traxler D, Kasiri MM, Hofbauer H, Wolzt M, Glogner C, et al. Safety and tolerability of topically administered autologous, apoptotic PBMC secretome (APOSEC) in dermal wounds: a randomized Phase 1 trial (MARSYAS I). *Sci Rep*. 2017;7:6216. doi:10.1038/s41598-017-06223-x.
62. Gugerell A, Gouya-Lechner G, Hofbauer H, Laggner M, Trautinger F, Almer G, et al. Safety and clinical efficacy of the secretome of stressed peripheral blood mononuclear cells in patients with diabetic foot ulcer-study protocol of the randomized, placebo-controlled, double-blind, multicenter, international phase II clinical trial MARSYAS II. *Trials*. 2021;22:10. doi:10.1186/s13063-020-04948-1.
63. Scatena A, Petruzzi P, Maioli F, Lucaroni F, Ambrosone C, Ventoruzzo G, et al. Autologous peripheral blood mononuclear cells for limb salvage in diabetic foot patients with no-option critical limb ischemia. *J Clin Med*. 2021;10:2213. doi:10.3390/jcm10102213.
64. Tanaka R, Ito-Hirano R, Fujimura S, Arita K, Hagiwara H, Mita T, et al. Ex vivo conditioning of peripheral blood mononuclear cells of diabetic patients promotes vasculogenic wound healing. *Stem Cells Transl Med*. 2021;10:895-909. doi:10.1002/sctm.20-0309.
65. Mildner M, Hacker S, Haider T, Gschwandtner M, Werba G, Barresi C, et al. Secretome of peripheral blood mononuclear cells enhances wound healing. *PLoS One*. 2013;8:e60103. doi:10.1371/journal.pone.0060103.
66. Kado M, Tanaka R, Arita K, Okada K, Ito-Hirano R, Fujimura S, et al. Human peripheral blood mononuclear cells enriched in endothelial progenitor cells via quality and quantity controlled culture

Figures

Figure 1

Proliferation and characteristic of the peripheral blood mononuclear cells-(PBMCs-) treated IronQ complex. (a) Representative phase-contrast images of the morphological states of PBMCs on day 10 of the culturing process. (b) PBMCs were cultured in the presence of 125 $\mu\text{g/mL}$ of the IronQ complex. Morphological observation at different time points revealed that IronQ increased the number of adherent cells by almost 90% confluence on Day 14. (c) PBMCs were cultured in the presence of 125 $\mu\text{g/mL}$ of the IronQ complex. CFU-Hill or early outgrowth EPCs were observed after 7 days of culturing. The white box areas in the upper images ($\times 40$) were magnified in the lower images ($\times 100$). Scale bar = 100 μm . (d) A proliferation assay over 10 days of culturing revealed that the cells generated under IronQ conditions showed greater proliferation. Data are presented as the mean $\pm$ SD; n = 8. * P < 0.05

Figure 2

Flow cytometry analysis of pre-and-post IronQ PBMCs. (a) Scatter diagrams of pre-and-post IronQ PBMCs in flow cytometry. The red lines indicate the cellular-sized gates of lymphocyte and monocyte (R1) or the larger cell (R2). (b) Flow cytometry analysis for stem cell (CD34), hematopoietic cells (CD14, CD11b, and CD45), and angiogenic (CD105, VEGFR-2, and CD31) markers in post-IronQ PBMCs (at day 10). (c) The bar graph shows the ratio of each percent (%) cell positivity in post-IronQ PBMCs (at day 10) to that in untreated PBMCs. The column represents a mean $\pm$ SD in each increase or decrease (N= 16), *P < 0.05. (d) Flow cytometric analysis of kinetic profiles of marker expression across population expansion. The data are presented as mean $\pm$ SD (n = 8). PBMCs, peripheral blood mononuclear cells; IronQ, Iron-Quercetin complex; VEGFR-2, vascular endothelial growth factor receptor 2.

Figure 3

Treatment of PBMCs with IronQ complex promotes the secretion of vasculogenic, anti-inflammatory, and wound healing factors. Enzyme-linked immunosorbent assay (ELISA) measurement of cytokines in untreated PBMCs-conditioned medium and IronQ-preconditioning PBMCs -conditioned medium (at day 10). Data are expressed as means $\pm$ SD (n = 8). PBMCs, peripheral blood mononuclear cells.

Figure 4

PBMCs treated IronQ exhibit pro-angiogenic potential on HUVEC tubular network. (a) Tube formation ability of HUVECs cultured in conditioned medium from PBMCs control (control PBMC-CM), condition medium from IronQ-preconditioning PBMCs (post-IronQ PBMC-CM), or basal medium for endothelial cells (EGM). Scale bar = 100 μm, FOV (field of view). (b) Coculture of HUVECs and PBMCs treated IronQ (green fluorescence label) on Matrigel. Green fluorescence and merge images at 4, 16, and 24 h are shown. Scale bar = 100 μm. (c, d) Quantitative evaluation of tube length and tube number after HUVECs cultured in conditioned medium from PBMCs; the tube length and tube number were calculated in four random fields. Data are expressed as means ± SD. * $P < 0.05$, ** $P < 0.01$.

Figure 5

The effect of IronQ-preconditioning PBMCs secreted factors on the migration of HUVECs. HUVECs were cultured in the upper chamber of the Transwell chambers with serum-free medium (0.1% FBS), and lower chamber were filled with 0.1% FBS medium (Negative control), 0.1% FBS + Control PBMC-CM, 0.1% FBS + post-IronQ PBMC-CM, or 10% FBS medium (Positive control). (a) After 24 h, the number of cells on the lower surface of the upper chamber was counted under an inverted microscope at $\times 100$ magnification. (b) Quantitation of HUVEC cells migration (violet stained cells) using a Transwell chamber. The quantitative evaluation of the number of cells migrated to the lower surface of Transwell chamber with different conditions after 24 h of the culture period. Data are expressed as means $\pm$ SD ($n = 8$). * $P < 0.05$

Figure 6

Effects of the conditioned medium by IronQ-preconditioning PBMCs on fibroblast migration in the wound scratch assay. (a) Representative images of scratch assays of L929 fibroblast immediately after the scratches had been made (0 h) and then after 6, and 9 h in the presence of PBMC-CM versus ordinary medium. Scale bar = 100 μ m. (b) Scratch closure was measured as the width of each scratch using Image J software. Each time point was normalized to the image perimeter on 0 h and reported as percent healing. The results are expressed as means $\pm$ SD (n = 8). * P < 0.05.